
Appendix J

Refractory Toxicity Assessment Protocol: Step-by-Step Procedures

The following protocol provides step-by-step procedures for designing and executing RTA studies to track sources of acute and/or chronic toxicity in POTW collection systems. This protocol describes the following steps:

- Using characterization data to evaluate waste streams of concern.
- Accounting for toxicity in the activated sludge biomass to be used in testing.
- Adapting and calibrating the protocol to site-specific conditions.
- Collecting and analyzing samples to be used in testing.
- Preparing RTA test mixtures.
- Performing RTA tests.
- Evaluating the inhibitory potential of waste streams.
- Performing TIE Phase I tests on RTA effluents (optional).

The RTA protocol was first developed in the USEPA TRE research study at the City of Baltimore's Patapsco POTW (Botts et al., 1987) to evaluate the potential for indirect dischargers to contribute refractory toxicity. Additional USEPA TRE research studies in Linden, New Jersey; High Point, North Carolina; Fayetteville, North Carolina; and Bergen County, New Jersey were conducted to improve the RTA approach (Morris et al., 1990; DiGiano, 1988; Fillmore et al., 1990; Collins et al., 1991). The RTA protocol described below is a refined version of the method given in the first edition of the Municipal TRE Protocol (USEPA, 1989).

The RTA procedure has been used to track sources of acute and chronic toxicity using both freshwater and

estuarine/marine species (Morris et al., 1990; Botts et al., 1992, 1993, 1994). Examples of RTA studies are presented in Appendices C, D, and G. The RTA protocol has been designed to simulate conventional activated sludge processes, although it has also been adapted to other POTW treatment processes including single and two-stage nitrification systems (Collins, et al. 1991), BNR processes (Appendix D), and filtration treatment systems (Appendices C and D).

A. POTW Wastewater Profile

Characterization data are generated for each waste stream to be tested in the RTA.

1. Collect grab samples of RAS and 24-hour composite samples of POTW primary effluent and selected sewer wastewaters (i.e., sewer line wastewater or indirect discharges).
2. Analyze RAS samples (filtrate) for TSS, VSS, $\text{NH}_3\text{-N}$, and pH.
3. Analyze primary effluent and sewer wastewater samples for BOD_5 , COD, TSS, TKN, TP, $\text{NH}_3\text{-N}$, and pH.
4. Determine the type of unit processes, type of discharge (e.g., continuous versus intermittent), operations schedule, and flow rate for the discharge points selected for evaluation (see Section 5).
5. Repeat above steps on several samples to characterize variability over time.

B. Biomass Toxicity Measurement

Biomass toxicity is measured to evaluate the potential for toxicity interferences in the RTA.

1. Collect 5 liters of fresh RAS and aerate vigorously for 15 minutes.
2. Prepare glass fiber filter [same type used for TSS analysis (APHA, 1995) by rinsing two 50 ml volumes of high purity water through the filter.
3. Filter sufficient volume of RAS for two acute or chronic toxicity tests.¹
4. Centrifuge a portion of the RAS filtrate at 10,000 xg for 10 to 15 minutes. Alternatively, filter RAS filtrate through a 0.2 µm pore-size filter if blank tests show that the filter does not remove soluble toxicity or add artificial toxicity (see Section 5).¹
5. Test RAS filtrate and RAS centrate/fine filtrate for acute toxicity using procedures described by USEPA (1991a, 1991b) or for chronic toxicity using limited-scale methods provided by USEPA (1992a, 1992b, 1992c, 1995, 1996).
6. Repeat above steps on several RAS samples to characterize variability over time.
7. If RAS filtrate is more toxic than the RAS centrate/fine filtrate, obtain non-toxic biomass (e.g., another POTW biomass or a freeze-dried preparation) (see Section 5).

C. RTA Reactor Calibration Testing²

Calibration tests are performed to select the RTA test operating conditions that most closely simulate the POTW operation and performance.

¹ Positive pressure filtering is recommended. Chronic toxicity measurement will require larger filtrate volumes than acute tests.

² RTA calibration is recommended. If resources are limited, POTW staff may select test conditions that reflect POTW operating conditions. However, RTA reactor performance should be compared to POTW performance to ensure that the RTA procedure effectively simulates the POTW processes.

1. As described in Section 5, estimate MLVSS concentration for RTA batch tests using mathematical models (Grady and Lim, 1980; Kornegay, 1970). Alternatively, use the average MLVSS concentration for the POTW.
2. Select a series of MLVSS concentrations (e.g., four) that includes the model MLVSS concentration. Calculate the volumes of RAS (V_r) needed to yield the MLVSS concentrations in the batch reactors. If the RAS was found to be toxic (i.e., RAS filtrate is more toxic than RAS centrate in step B-5 above), also select appropriate volumes of non-toxic biomass (V_{nb}). An equation for calculating V_b and V_{nb} is:

$$V_b \text{ or } V_{nb}(\text{L}) = \frac{\text{Target MLVSS (mg/L)}}{\text{RAS VSS (mg/L)}} \times V_r(\text{L}),$$

where: V_r is the reactor test volume.

3. Add each RAS volume (V_b and V_{nb}, if needed) to pre-cleaned glass or clear plastic containers. Add diffused air using air stones and gently aerate. Note that it may be necessary to filter the air supply to prevent contamination (e.g., compressor oil) of the reactor mixed liquors.
4. Add primary effluent (V_{pe}) to each reactor containing V_b and V_{nb}. V_{pe} can be calculated using the following equation:
$$V_{pe} = (V_r - V_b).$$
5. Adjust aeration rate to maintain DO at concentrations typically observed in POTW activated sludge process. Mechanical mixing using a magnetic stirrer and teflon-coated stir bars may be required to ensure complete mixing. Periodically check and adjust DO level.

6. Periodically check the batch reactor pH. Adjust pH to 6–9 range, if necessary.
7. Periodically collect 50–100 ml samples of batch reactor mixed liquor from each reactor (e.g., 1- to 2-hour intervals).
8. Allow mixed liquor samples to settle for 15 minutes. Rinse glass fiber filters as stated in

step B-2 above. Filter each mixed liquor supernatant using separate filters.

9. Stop aeration after the required reaction period, allow the Vb (and Vnb) to settle for 15 minutes, and filter the clarified batch effluents as described in step C-8.
10. Analyze filtered batch mixed liquor and effluent samples to determine COD removal over time.
11. Decant additional clarified batch effluent for toxicity analysis. Filter each batch effluent using rinsed filters.¹ Wash filter apparatus between each sample filtration using high-purity water.
12. Batch filtrates that were treated with toxic biomass (Vb) must be centrifuged at 10,000 xg for 10 to 15 minutes to remove colloidal size particles. Viscous mixtures may require faster or longer centrifugation (ASM, 1981). Alternatively, the batch filtrates may be filtered through a 0.2 µm pore-size filter if the filter does not remove soluble toxicity (see Section 5).³ Filter blank analyses should be performed for each filter type using high-purity water.
13. Analyze the batch effluent filtrates, centrates, and filter blanks for acute or chronic toxicity using the procedures referenced in step B-5.
14. Calibration test results can be used to select a batch MLVSS concentration that achieves a level of COD and toxicity removal similar to that provided by the POTW activated sludge process (see Section 5).

D. Sample Collection

Representative samples are collected from each waste stream to be tested in the RTA.

1. Upon completion of the RTA calibration, tests can be conducted to evaluate the refractory toxicity of sewer wastewaters.

2. Obtain 24-hour, flow-proportioned composite samples of sewer wastewater (i.e., sewer line wastewater or indirect discharger effluent) and POTW primary effluent. If possible, lag collection of the primary effluent sample by the estimated travel time of the sewer wastewater to the POTW.
3. Collect 10 liters of RAS (and non-toxic biomass, if needed) on day of test.

E. Sample Characterization (performed on day of sample collection)

Sample characterization data are collected to set the operating conditions for the RTA.

1. Analyze sewer wastewater for BOD₅, COD, TSS, TKN, TP, NH₃-N, and pH.
2. Prepare glass fiber filter as stated in step B-2. Filter RAS and test filtrate for acute or chronic toxicity using the procedures referenced in step B-5.⁴
3. Determine percent volume of sewer wastewater in POTW influent based on flow data gathered in the wastewater profile (step A above).

F. Preparation of RTA Test Mixtures

Two types of batch reactors are prepared: one consisting of the POTW influent (primary effluent) and RAS, which serves as a control, and another consisting of the sewer wastewater spiked into the POTW influent and RAS.

1. Calculate the volume of sewer wastewater (Vw) based on the sewer wastewater flow and the desired flow concentration factor (Fw). Information on selecting an appropriate Fw is presented in Section 5. Vw can be calculated using the following equation:

$$V_w(L) = \frac{Q_w}{Q_i} \times (V_r - V_b) \times F_w,$$

³ Positive pressure filtering is recommended. Also, chronic toxicity measurement will require larger filtrate volumes than acute toxicity tests.

⁴ Positive pressure filtering is recommended. Also, chronic toxicity measurement will require larger filtrate volumes than toxicity tests.

where: Q_w is the sewer wastewater flow rate (mgd).

Q_i is the average POTW influent flow rate (mgd).

F_w is the sewer wastewater flow concentration factor (e.g., 1, 2, 10 times the sewer wastewater flow).

2. Calculate the volume of primary effluent (V_{pe}) using the following equation:

$$V_{pe} = (V_r - V_b - V_w).$$

3. Prepare spiked batch reactor influent by mixing V_w with V_{pe} and measure V_{pe} for control batch reactor influent.
4. If necessary, add nutrients to adjust the $BOD_5/TKN/TP$ ratio of the spiked batch influent to equal the average $BOD_5/TKN/TP$ ratio of the POTW influent (or 100:5:1). An equation for calculating BOD_5 , TKN, and TP concentrations in the spiked batch influent is:

$$BOD_5, TKN, \text{ or } TP (C, \text{mg/L}) = \frac{(V_{pe} \times C_{pe}) + (V_w \times C_w)}{(V_{pe} + V_w)},$$

where: C_{pe} is the BOD_5 or nutrient concentration in primary effluent (mg/L).

C_w is the BOD_5 or nutrient concentration in sewer wastewater (mg/L).

5. If necessary, adjust pH of batch influents to pH range for POTW influent.
6. Test sample toxicity (using methods referenced in step B-5) after nutrient addition and pH adjustment to determine if the batch influent toxicity is changed by these steps.
7. Select volume of RAS (V_b) to yield the MLVSS concentration determined in calibration testing (step C above). If RAS is toxic (i.e., RAS filtrate is more toxic than RAS centrate), also select appropriate volume of non-toxic biomass (V_{nb}). The equation for calculating V_b and V_{nb} is provided in step C-2.

8. Add each RAS volume (V_b and V_{nb} , if needed) to pre-cleaned glass or clear plastic containers.

9. Add spiked batch influent and control batch influent to reactors containing V_b (and reactors containing V_{nb} , if needed).

G. Performance of RTA Tests

The spiked batch reactor influent and control batch reactor influent are treated and the resulting effluents are tested for toxicity.

1. Add diffused air to reactors using air stones and gently aerate. Note that it may be necessary to filter the air supply to prevent contamination (e.g., compressor oil) of the reactor mixed liquors.
2. Adjust aeration rate to maintain DO at concentrations typically observed in the POTW activated sludge process. Mechanical mixing may be required to ensure complete mixing. Periodically check and adjust the DO level.
3. Periodically check the batch reactor pH and adjust to pH 6-9 range, if necessary.
4. The treatment period for the control reactor should be equal to the average HRT of the POTW aeration system. For the spiked reactor, calculate the required reaction period necessary to achieve a batch F/M ratio (F/M_b) equal to the nominal F/M ratio determined in calibration testing (step C above). F/M_b can be calculated using the following equation:

$$\text{Test Period (days)} = \frac{\text{Batch Influent COD (mg/L)}}{(\text{MLVSS (mg/L)} \times F/M_b)},$$

where: F/M_b is equal to the calculated F/M of the control (primary effluent) reactor.

$F/M_b = \text{COD}_{pe} / (\text{MLVSS} \times \text{test period, days}).$

5. Stop aeration after the required reaction period and allow the Vb (and Vnb) to settle for 1 hour. Decant the clarified batch supernatant for toxicity analysis. Filter each batch supernatant using rinsed filters.⁵ Wash filter apparatus between each sample filtration using high-purity water.
6. Batch filtrates that were treated with toxic biomass (Vb) must be centrifuged at 10,000 xg for 10 to 15 minutes to remove colloidal size particles (ASM, 1981). Alternatively, the batch filtrates may be filtered through a 0.2 µm pore size filter if the filter does not remove soluble toxicity (see Section 5).¹ Filter blank analyses should be performed for each filter type using high-purity water.
7. Analyze the batch filtrates, centrates, and filter blanks for acute or chronic toxicity using the procedures referenced in step B-5 above.

H. Synthetic Wastewater Testing (Optional)

Synthetic wastewater can be used in lieu of POTW influent (primary effluent) in the RTA to determine the toxicity of the sewer wastewater.

1. Select non-toxic synthetic wastewater. Confirm that the synthetic wastewater is non-toxic using toxicity test procedures referenced in step B-5 above.
2. Prepare synthetic wastewater solution with SCOD concentration equal to the average SCOD of the POTW primary effluent.
3. Prepare volume of synthetic wastewater (Vsw) equal to the volume of primary effluent (Vpe) used above for the sewer wastewater/primary effluent batch test.
4. Add Vw and Vsw to a reactor containing Vb (and a reactor containing Vnb, if needed).
5. After batch treatment, analyze batch effluent toxicity as described in step G above.

I. Inhibition Testing (Optional)

The RTA protocol can be used to evaluate the inhibitory potential of the sewer wastewater.

1. Add equal volumes of Vb to four reactors. Add diffused air and gently aerate.
2. Prepare a series of four sewer wastewater concentrations (e.g., 100, 50, 25 and 12.5% wastewater) by adding sewer wastewater to toxicity test dilution water (freshwater).
3. If necessary, add nutrients to adjust batch influent BOD₅/TKN/TP ratio.
4. Add sewer wastewater volumes (e.g., Vw100, Vw50, Vw25 and Vw12.5) to the reactors.
5. Adjust aeration rate to maintain DO at concentrations typically observed in the POTW activated sludge process. Mechanical mixing may be necessary to ensure complete mixing. Periodically check and adjust DO level.
6. Periodically check the batch reactor pH and adjust to pH 6–9 range, if necessary.
7. Subsample 300 ml from each reactor at 30 minutes and every 2 hours following test initiation. Immediately measure oxygen utilization using the BOD bottle method (APHA, 1995). Return the subsamples to the reactors immediately following oxygen utilization measurement. Alternatively, oxygen utilization can be measured using respirometric techniques.
8. Subsample 50 ml from each reactor at 5 minutes and every 2 hours following test initiation, and at completion of the test. Also, subsample 50 ml of the original undiluted RAS. Filter the subsamples through a 0.45 µm pore-size filter and measure the SCOD of the filtrates.
9. Calculate oxygen and COD utilization rates, as described in Section 5 of this manual, and plot rates versus sewer wastewater concentration. Lower oxygen and COD removal rates with increasing wastewater concentration may indicate inhibition.

⁵ Positive pressure filtering is recommended. Also, chronic toxicity measurement will require larger filtrate volumes than acute toxicity tests.

J. Phase I Toxicity Characterization (Optional)

1. TIE Phase I tests may be conducted on RTA test effluents using indirect discharger wastewater spiked into primary effluent. Additional volumes are required for TIE Phase I testing; therefore, the batch reactor volume will need to be increased accordingly (USEPA 1991a, 1992a, 1996).
2. TIE Phase I tests should be performed on effluent filtrates from RTA tests that use non-toxic POTW biomass.

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